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Insights into the molecular mechanism of action of *Celastraceae* sesquiterpenes as specific, non-transported inhibitors of human P-glycoprotein

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Abstract

Dihydro-β-agarofuran sesquiterpenes from *Celastraceae* have been recently shown to bind to human P-glycoprotein (Pgp), functioning as specific, mixed-type inhibitors of its drug transport activity, as well as multidrug resistance (MDR) modulators in vitro. However, nothing is known about whether such compounds are themselves transported by Pgp, or whether they affect Pgp expression as well as its activity, or about the location of their binding site within the protein. We performed transport experiments with a newly synthesized fluorescent sesquiterpene derivative, which retains the anti-Pgp activity of its natural precursor. This probe was poorly transported by Pgp, MRP1, MRP2 and BCRP transporters, compared with classical MDR substrates. Moreover, Pgp did not confer cross-resistance to the most potent dihydro-β-agarofurans, which did not affect Pgp expression levels in several MDR cell lines. Finally, we observed competitive and non-competitive interactions between one of such dihydro-β-agarofurans (Mama12) and classical Pgp modulators such as cyclosporin A, verapamil, progesterone, vinblastine and GF120918. These findings suggest that multidrug ABC transporters do not confer resistance to dihydro-β-agarofurans and could not affect their absorption and biodistribution in the body. Moreover, we mapped their binding site(s) within Pgp, which may prove useful for the rational design of improved modulators based on the structure of dihydro-β-agarofurans.

Keywords: P-glycoprotein; Dihydro-β-agarofurans; Fluorescent derivative; Fluorescent dye extrusion; Binding site mapping

1. Introduction

A considerable number of cancers are either intrinsically resistant or exhibit treatment-induced acquired resistance, which complicates efforts for the successful treatment of cancer. Expression of P-glycoprotein MDR1 (Pgp) is among the best documented mechanisms of MDR (multidrug resistance) in human clinical cancers [1], and it has been detected in virtually all tumour types in man at both diagnosis and during relapse [2–4]. Moreover, expression of Pgp in many human cancers correlates with response to therapy and survival [5–7]. The use of Pgp modulators has been suggested as a way to overcome Pgp-mediated MDR [8] or even to prevent the emergence of MDR in cancer patients [9]. Many agents that modulate Pgp have been identified since the 1980s [10], but most of them yielded disappointing results in vivo because they were themselves transported by Pgp, competing with the cytotoxic compounds being effluxed by the pump. Therefore, they were too weak as Pgp inhibitors and very high serum levels were necessary to achieve effective MDR reversion in patients, resulting in unacceptable toxicity [11]. Moreover, many of these chemosensitizers lack specificity, inhibiting also other ABC transporters with well-defined physiological roles related to

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regulation of absorption, distribution and clearance of xenobiotics [12,13], leading to greater side effects of anticancer drugs and resulting in unpredictable pharmacokinetic interactions that preclude their clinical use. Therefore, a Pgp modulator for potential clinical use must be sufficiently effective to inhibit Pgp (must itself be non-transportable), must be devoid of intrinsic toxicity, and highly specific for Pgp to avoid pharmacological interactions with the co-administered anticancer drugs [14].

In our previous work, we demonstrated that dihydro-β-agarofuran sesquiterpenes from *Celastraceae* family of plants bind to transmembrane domains (TMDs) of human Pgp MDR1 (ABCB1), functioning as potent and specific mixed-competitive inhibitors of its drug transport activity, with insignificant inhibitory effects on the multidrug efflux pumps MRP1 (ABCC1), MRP2 (ABCC2) and BCRP (ABCG2) [15]. However, it remained to be determined if these natural products are themselves transported by ABC multidrug efflux pumps, if they alter Pgp expression levels as well as its activity, and besides, we wanted to map their putative binding site within the Pgp TMDs. For the former purpose, we have synthesized a new fluorescent sesquiterpene derivative, which retains most of the MDR reversal activity of its natural precursor. By using such a derivative in transport studies, we have demonstrated in situ that dihydro-β-agarofurans are either transported poorly or not at all by Pgp or any other ABC transporter known to be involved in cancer MDR. Furthermore, Pgp does not confer cross-resistance to the most potent natural sesquiterpenes isolated in our lab (Mama5, Mama12 and Machu4) [15], which supports the finding described above. In addition, these natural sesquiterpenes do not affect Pgp expression levels in a series of MDR cell lines. Finally, we measured the effects of individual Pgp modulators and pairs of modulators on the accumulation of daunorubicin (DNM) in NIH-3T3 MDR1 cells, and performed a simple kinetic analysis that enables such data to be modelled in terms of competitive, co-operative or non-competitive interaction between pairs of modulators. Mama12, one of the most potent Pgp sesquiterpene inhibitors described to date, seems to possess two different binding sites, of high and low affinity, and at least one of them appears to interact competitively with that of cyclosporin A and verapamil. Moreover, Mama12 does not bind to vinblastine-binding site(s), or to progesterone-binding sites [both in TMDs and in nucleotide binding domains (NBDs)]. Finally, Mama12 cooperates in a synergistic manner with elacridar (GF120918) to bring about reversal of Pgp. The information about the manner in which dihydro-β-agarofurans bind to Pgp and interact with other Pgp substrates and modulators could be of importance for the rational design of new, improved Pgp inhibitors based on the molecular structure of dihydro-β-agarofurans.

2. Materials and methods

2.1. Chemicals

Vinblastine (VNB), G418, adriamycin, vincristine, verapamil, cyclosporin A (CsA), progesterone (PRG), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium], colchicine, *N*-methylisatoic anhydride (MIA), *N*-*N*-dimethylformamide (DMF), and 4-dimethylaminopyridine (DMAP) were from Sigma-Aldrich. [^{3}H]-Azidopine (52 Ci/mmol) and NAMP100 amplifying solution for fluorography were from Amersham Biosciences. Calcein-AM and Hoechst 33342 were from Molecular Probes. GF120918 was from GlaxoSmithKline. The C219 monoclonal antibody, directed against Pgp, was from Calbiochem. Daunorubicin (DNM) was from Pfizer. Natural dihydro-β-agarofuran sesquiterpenes from *Celastraceae* plants Mama5, Mama12 and Machu4 were isolated, purified and characterized as described previously [16]. The sesquiterpene [1α-acetoxy-9α-benzoyloxy-8β,15-di(2-methylbutyroyloxy)-2α,4β,6β-trihydroxydihydro-β-agarofuran] from *Crossopetalum tonduzi* (sesquiterpene T3), described previously [17], was used for the synthesis of the 6β-methylanthranoyl (MANT) fluorescent derivative (excitation/emission wavelength ~350/446 nm) (Fig. 1).

2.2. Synthesis of MANT-sesquiterpene

In order to synthesize a fluorescent dihydro-β-agarofuran derivative active as a Pgp modulator, the following considerations were taken into account: firstly, the parental sesquiterpene should be itself active against Pgp and possess a free hydroxyl group at position C-6 to allow the acylation of the molecule with the chosen fluorescent probe. Secondly, the chosen fluorescent probe to be attached at C-6 should resemble in size and chemical nature that of the typical groups that occur in the basic scaffold of active natural sesquiterpenes. For these reasons, sesquiterpene T3 and MIA were chosen as the parental molecule and the fluorescent probe precursor, respectively.

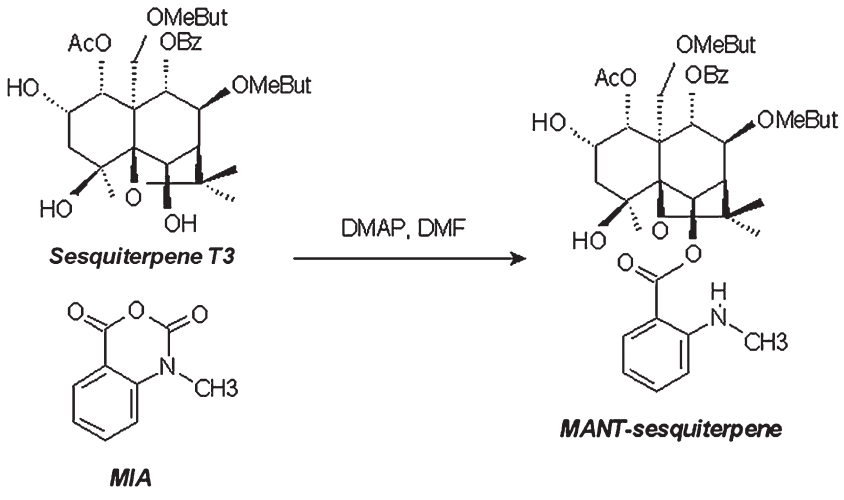

Fig. 1. Synthesis of the fluorescent derivative MANT-sesquiterpene. The synthesis was achieved by regioselective acylation with MIA of the hydroxyl group at C-6β of the natural sesquiterpene 1α-acetoxy-9α-benzoyloxy-8β,15-di(2-methylbutyroyloxy)-2α,4β,6β-trihydroxydihydro-β-agarofuran (*T3*).

Attachment of the fluorescent group MANT (methylantranoyl) to the above-mentioned natural dihydro-β-agarofuran sesquiterpene T3 was achieved by regioselective acylation of the hydroxyl group at C-6β with MIA. Optical rotations were measured on a Perkin-Elmer 241 automatic polarimeter, and the $[\alpha]_D$ are given in 10^{-1} deg cm^2 g^{-1}. IR (film) spectra were recorded on a Bruker IFS 55 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 300 spectrometer. Electron impact mass spectroscopy (EIMS) and high-resolution EIMS (HREIMS) were recorded on a Micromass Autospec spectrometer. Monitoring and purification of the reactions were performed using silica gel (TLC, plastic sheets, silica gel 60–250 UV_{250}, Panreac).

MIA, DMF and DMAP were used without further purification. A solution of sesquiterpene T3 (6.0 mg, 9.3×10^{-3} mmol), MIA (4 mg, 2.2×10^{-3} mmol) and DMAP (0.2 mg, 1.6×10^{-3} mmol) in dry DMF (1.0 ml) was stirred for 18 h at room temperature. The reaction was quenched by the addition of ethanol (0.5 ml) followed by stirring for 30 min at room temperature. The mixture was evaporated with dryness, and the residue was purified by preparative TLC using n-hexane/ethyl acetate (1:1), yielded 2.6 mg (36%) of the desired MANT-sesquiterpene as a colourless lacquer, followed by 2.8 mg (47%) of the starting material: $[\alpha]_D^{20}=+40°$ (*c* 0.16, $CHCl_3$); IR γ_{max} (film) 3525, 3385, 2960, 2925, 2854, 1732, 1681, 1580, 1368, 1263, 1239, 1154, 1094, 1031, 755, 712 cm^{-1}; HNMR ($CDCl_3$) δ 0.58 (3H, t, *J*=7.4 Hz), 0.87 (3H, d, *J*=7.0 Hz), 0.95 (3H, t, *J*=7.4 Hz), 1.33 (3H, d, *J*=6.8 Hz), 1.38 (3H, s), 1.56 (3H, s), 1.64 (3H, s), 1.71 (3H, s), 1.82 (2H, m), 2.08 (2H, m), 2.21 (1H, m), 2.71 (1H, d, *J*=3.4 Hz), 2.77 (1H, m), 2.91 (3H, d, *J*=5.0 Hz), 4.16 (1H, d, *J*=2.7 Hz), 4.86, 5.06 (2H, d_{ab}, *J*=13.4 Hz), 5.49 (1H, d, *J*=2.9 Hz), 5.84 (1H, dd, *J*=3.4, 9.8 Hz), 6.14 (1H, d, *J*=9.8 Hz), 6.50 (1H, s), 6.63 (2H, m), 7.45 (3H, m), 7.57 (1H, d, *J*=7.6 Hz), 7.70 (1H, d, *J*=5.6 Hz), 7.94 (2H, dd, *J*=1.2, 8.3 Hz), 8.18 (1H, dd, *J*=1.4, 7.9 Hz); ^{13}C NMR ($CDCl_3$) δ 11.1 (q), 11.7 (q), 16.1 (q), 16.8 (q), 21.0 (q), 24.7 (q), 25.8 (q), 26. 2 (t), 26.6 (t), 29.5 (q), 31.9 (q), 41.1 (d), 41.6 (d), 44.3 (t), 51.7 (s), 52.1 (d), 62.1 (t), 67.5 (s), 69.9 (d), 73.8 (d), 75.6 (d), 84.0 (s), 78.1 (d), 92.2 (s), 109.5 (s), 110.7 (d), 114.9 (d), 128.6 (2x d), 129.6 (2x d, s), 132.7 (d), 133.4 (d), 135.0 (d), 152.0 (s), 165.9 (s), 167.2 (s), 169.7 (s), 175.2 (s), 176.3 (s); EIMS *m*/*z* (%) 781 (M^+, 0.5), 691 (1.0), 675 (0.7), 663 (0.5), 569 (0.7), 394 (1), 369 (0.8), 322 (2.9), 283 (0.6), 250 (1.1), 221 (2.0), 191 (1.2), 136 (14.7), 109 (20.1), 81 (37.5), 57 (100); HREIMS *m*/*z* calcd for $C_{42}H_{55}N_1O_{13}$ 781.36734, found 781.36710.

2.3. Cell cultures

Mammalian cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO_2, with their respective media supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 250 U/ml penicillin G plus 250 μg/ml streptomycin sulphate (Invitrogen), as well as the appropriate concentration of drugs indicated. The cell lines were: parental, drug-sensitive NIH-3T3 cells and their MDR counterpart transfected with human MDR1-G185 (cultured in the presence of colchicine 60 ng/ml) [18]; parental, drug-sensitive AuxB1 and the MDR colchicine-selected lines CH^{R}C5 (colchicine 10 μg/ml) [19], and CH^{R}B30 (colchicine 30 μg/ml) [20]; parental, drug-sensitive MDCKII cells and their MDR counterpart transfected with human MRP2 [21]; parental 2008 cells and their MDR counterpart transfected with human MRP1 [22]; parental MDA-MB-231 cells and their MDR counterpart transfected with human BCRP, MDA-MB-231 BCRP clone F12 (G418 400 μg/ml), obtained in our laboratory by subcloning of the original clone 23 [23] by the limit dilution method; parental, drug-sensitive L1210S cells and the MDR L1210R (DNM 1 μM) [24]; parental CCRF-CEM cells and the MDR sublines CCRF/ADR5000 (adriamycin 5 μg/ml) and CCRF/VCR1000 (vincristine 1μg/ml) [25].

2.4. Cytotoxicity assays with sesquiterpenes and their influence on Pgp expression

Intrinsic toxicity of sesquiterpenes in drug-sensitive cells and their Pgp-expressing counterparts, as well as reversion of resistance to DNM and VNB, were determined by the MTT colorimetric assay in 96 well-plates as described [15]. Dose–response curves were generated by plotting percentage of cell growth against concentration of cytotoxic drug (DNM or VNB), and the data points (mean±S.D.; $P<0.05$) were fitted by non-linear regression to a four parameters logistic curve using SigmaPlot 2000 for Windows (SPSS Inc.) to determine IC50 values and to calculate reversal indexes (ratio between IC50 without and with modulator). The influence of sesquiterpenes Mama5, Mama12 and Machu4 on Pgp expression was determined by measuring the expression levels of the protein in CH^{R}C5, CH^{R}B30, L1210R, CCRF/ADR5000 and CCRF/VCR1000 cells after 72 h incubation in the presence of 3 μM of each sesquiterpene. We checked first that such a concentration was able to sensitize MDR cells by 60–90%, depending on the cell line, with low intrinsic toxicity. After this incubation period, cells were harvested, washed once with ice-cold PBS, diluted in ice-cooled hypotonic lysis buffer (10 mM Tris–HCl pH 7.4 containing a cocktail of protease inhibitors; Sigma-Aldrich) at a concentration of 1×10^6 cell/ml, and disrupted by 50 strokes in a teflon-glass homogenizer, monitoring the disruption process under the microscope. Then, the homogenates were centrifuged once at 500×*g* 4 °C to remove intact cells and nuclei, and the supernatant submitted to ultracentrifugation (100,000 × *g* 4 °C) to obtain the whole membrane and microsomal fraction. The protein concentration in each sample was measured by the method of Bradford, in order to process the same amount of total proteins of each sample and allow reliable comparison of Pgp expression among different samples. Proteins were resolved by SDS-PAGE on 9% gels and transferred to PVDF membranes (Millipore), to perform Western blot for immunodetection of Pgp using C219 monoclonal antibody.

2.5. MDR reversal and photoaffinity labeling assays with MANT-sesquiterpene

In order to check the anti-Pgp activity of the newly synthesized fluorescent sesquiterpene derivative, both the parental sesquiterpene (T3) and MANT-sesquiterpene were studied in parallel as follows: MTT assays were performed to test their respective abilities to reverse cellular resistance (as described above), as well as flow cytometry analysis to test the inhibition of Pgp-mediated DNM efflux in NIH-3T3 MDR1-G185 cells, as previously described [15]. Measurement of the fluorescence signal of individual cells due to DNM accumulation was performed with a Becton Dickinson FacScan. Moreover, in order to demonstrate that the MANT-sesquiterpene binds to Pgp as do natural sesquiterpenes, a competition assay of the photoaffinity labeling of Pgp by ^{3}H-azidopine was performed in the presence of a 100-fold molar excess of MANT-sesquiterpene, as described in [26], using CH^{R}B30 plasma membrane vesicles purchased from the laboratory of Dr. Frances J. Sharom (Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario, Canada).

2.6. Accumulation and efflux experiments with MANT-sesquiterpene

Intracellular accumulation experiments with the MANT-sesquitepene were performed by plating NIH-3T3 MDR1-G185, CH^{R}C5, 2008-MRP1, MDCKII-MRP2 or MDA-MB-231 BCRP clone F12 cells in 96-well plates (NUNC) at a density of $2–4\times10^4$ cells/well. The following day, when cells were forming a 90% confluent monolayer in all the wells of a plate, the old medium was removed and Pgp-, MRP1-, MRP2- and BCRP-expressing cells were incubated at 37 °C in the presence of 10 μM MANT-sesquiterpene at increasing times ranging from 0 to 180 min in order to generate time-dependent accumulation curves. For comparison, similar curves were also generated for the different cell lines in the presence of Pgp substrate DNM 10 μM (NIH-3T3 MDR1 and CH^{R}C5), 1 μM calcein-AM (2008-MRP1 and MDCKII-MRP2) or 1 μM Hoechst 33342 (MDA-MB-231 BCRP clone F12). After each incubation period, the monolayers were washed twice with cell culture medium plus 10% FBS in order to remove any extracellular trace of fluorescent MDR substrate or MANT-sesquiterpene. Finally, 50 μM of lysis buffer (Tris–HCl 20 mM pH 7.4, SDS 0.2%) were added to each well and incubated overnight at 4 °C in the dark. The accumulated intracellular fluorescence due to DNM ($\lambda_{excitation}$=480 nm; $\lambda_{emission}$=590 nm), calcein ($\lambda_{excitation}$=488 nm; $\lambda_{emission}$=520 nm), Hoechst 33342 or MANT-sesquiterpene ($\lambda_{excitation}$=350 nm; $\lambda_{emission}$=446 nm) was measured in a microplate spectrofluorometer "SpectraMax Gemini EM" (Molecular Devices) using SoftmaxPro 4.3 software (also from Molecular Devices).

Efflux experiments were performed as follows: cell monolayers in 96-well plates were incubated for 2 h at 37 °C in the presence of 10 μM MANT-sesquiterpene or DNM in order to load cells with each fluorophore. Afterwards, the fluorophore-containing medium was removed, the monolayers were washed

twice with cell culture medium plus 10% FBS, and 100 μl of either normal medium plus 10% FBS (to measure the intracellular retained fluorescence only) or HPMI-glucose medium (to measure both intra and extracellular fluorescence), both without fluorophore, were added to wells and incubated for different times to allow the fluorophores to be effluxed out of cells. After different incubation periods ranging from 0 to 90 min, the samples containing normal medium plus 10% FBS were submitted to another washing before adding the lysis buffer, in order to remove extracellular fluorescence and to measure only the remaining intracellular fluorescence. On the other hand, lysis buffer was added directly to the samples containing HPMI-glucose medium without prior washing, and the entire fluorescence signal was measured, i.e., the sum of both the intracellular and the extracellular (effluxed) fluorescence. This control allowed us to rule out the possibility of a decrease in intracellular fluorescence due to metabolic degradation of the fluorescent probes, rather than active Pgp-mediated efflux from the cells. After lysis of cell monolayers, the fluorescence was measured as mentioned above and time-dependent efflux curves were generated with the resulting data points.

2.7. Fluorescence microscopy

Cells incubated with 1 μM MANT-sesquiterpene for 2 h at 37 °C were examined without previous fixation on a Zeiss Axiophot epifluorescence microscope. Images were captured with a SPOT camera (Diagnostic Instruments) and analysed using Adobe Photoshop 5.5 software.

2.8. Mapping of dihydro-β-agarofurans binding site within TMDs of Pgp

The effects were measured of combinations of verapamil, VNB, CsA, GF120918 (elacridar), PRG and natural sesquiterpene Mama12, used in pairs, on the accumulation of DNM into NIH-3T3 MDR1 cells as described above: cells plated in 96-well plates were exposed to 10 μM DNM in the absence/presence of modulators. When present, each modulator was used at increasing concentrations, either alone, or in combination with a different fixed concentration of a second modulator, in order to study how this second modulator affected the behaviour of the first modulator as a blocker of Pgp-mediated DNM efflux. Data analysis to establish the relationships among pairs of modulators were performed as described by You-ming Shao and coworkers [27]. Briefly, the authors used a simple kinetic analysis consisting in plotting the data of DNM inhibition experiments and fitting such data points to hyperbolic curves described by the equation

$$D_i = D_0 + (D_\infty - D_0) \times C^h / [K_i^h \times (D_\infty / D_0) + C^h], \quad (1)$$

where D_i is the amount of intracellular fluorescence due to DNM accumulation in cells at any concentration of modulator C. D_0 is DNM accumulation in the absence of modulator, D_∞ the accumulation at an infinitely high modulator concentration, K_i is the *intrinsic* inhibition parameter of the reverser (i.e., the concentration of modulator that produces 50% of DNM accumulation), and h is the Hill coefficient that determines the sigmoidicity of the curve.

The parallel equation (Eq. (2)) describes the effects of pairs of modulators in terms of the above variables and of the concentration of a second modulator B, which acts as a competitive modulator of the first one C. According to the classic competition model of the enzymologists, we have

$$D_i = D_0 + (D_\infty - D_0) \times C^h / [K_i^h \times (D_\infty / D_0) \times (1 + B) + C^h]. \quad (2)$$

Therefore, the effect of a competitive second modulator is to increase the apparent K_i of the first modulator by the factor $(1+B)$, where B is the concentration of the second modulator, divided by its intrinsic K_i. For the case of a synergistic second modulator, i.e., one that interacts non-competitively with the first modulator, the Eq. (1) remains unchanged. Such a non-competitive modulator will not affect the K_i of the first modulator. By fitting these equations to the data of D_i against C, at any value of B, using standard curve-fitting procedures, the appropriate values of K_i, D_0 and D_∞ can be found. From the dependence of this computed value of K_i (which refers to C) on the concentration of the second modulator B, one can test whether the interaction between the two modulators is additive (when K_i will increase with concentration of B) or synergistic (when K_i will remain unchanged). In the former case, one can find the appropriate value of K_i for the second reverser.

Furthermore, the drop in the Hill number, found in some cases as one increases the concentration of a second modulator, was used as a test for distinguishing between true synergism (between non-competitors) and the type of synergism displayed by two co-operating modulators, i.e., where the two different modulators can replace each other at the binding site of Pgp. To further support the above-described analysis, where necessary (see interaction between PRG and Mama12), we also determined the type of Pgp inhibition caused by pairs of modulators using the "Exploratory Enzyme Kinetics" application of the SigmaPlot 2000 software for Windows, whose enzymological basis is the direct linear plot [28]. By determining the type of interaction between sesquiterpene Mama12 and other Pgp modulators, we were able to map the sesquiterpene-binding site(s) of Pgp, and establish if that binding site is exclusive to sesquiterpenes, or whether it is shared by different modulators along with agarofurans.

3. Results

3.1. MANT-sesquiterpene binds to and inhibits drug transport activity of Pgp

The fluorescent derivative MANT-sesquiterpene should retain the MDR reversal ability of its natural precursor if it is intended to be a reliable probe for studying the mechanism of action of dihydro-β-agarofurans as Pgp inhibitors. Consequently, we studied the MDR reversal activity and the ability to block the Pgp-mediated DNM transport activity of both parental sesquiterpene and MANT-sesquiterpene in NIH-3T3 cells overexpressing human Pgp.

The ability of parental sesquiterpene (T3) (Fig. 1) and MANT-sesquiterpene to reverse cellular MDR to DNM and VNB in NIH-3T3 MDR1 cells was tested by MTT-based cytotoxicity assays. IC50 for DNM were 30.3±11.3 and 383.0±62.1 ng/ml in drug-sensitive and MDR cells, respectively (~12.8-fold resistance). On the other hand, IC50 for VNB in drug-sensitive and MDR cells were, respectively, 1.3±0.6 and 121.0±24.3 ng/ml (~93-fold resistance). An important MDR reversion, comparable to that caused by the classical modulator verapamil, was observed when adding sesquiterpene T3 to the cell culture medium: when reversing resistance to DNM, 3 and 10 μM T3 caused an IC50 shift in MDR cells from its original value (383.0±62.1 ng/ml) to 118.2±24.1 and 19.8±6.3 ng/ml, respectively (3.2- and 19.3-fold sensitization); on the other hand, when reversing VNB resistance, 3 and 10 μM T3 shifted IC50 from 121.0±24.3 ng/ml to 22.2±4.5 and 4.3±2.2 ng/ml, respectively (5.5- and 28.1-fold sensitization).

Regarding MANT-sesquiterpene, despite its chemical modification with respect to the parental molecule, it was also able to cause an important MDR reversion: at 3 and 10 μM, it was able to produce, respectively, 3.6- and 10.4-fold sensitization to DNM, and 1.9- and 12.3-fold sensitization to VNB. This means that MANT-sesquiterpene retains a significant ability to reverse MDR, only slightly affected by the MANT group.

In flow cytometry experiments, the fluorescence excitation and emission spectra of MANT-sesquiterpene and DNM were sufficiently different for fluorescence interference to exist between the two fluorophores, which could affect the results

of Pgp-dependent DNM transport inhibition experiments. These results are shown in Fig. 2A, demonstrating that MANT-sesquitepene inhibits DNM extrusion by Pgp with a potency similar to that of parental sesquiterpene and that of the classical modulator verapamil, at 1 and 10 μM.

Moreover, to confirm that MANT-sesquiterpene binds to Pgp, as was previously demonstrated for natural sesquiterpenes [15], we also performed competition assays of the photoaffinity labeling of Pgp by ^{3}H-azidopine in the presence of MANT-sesquiterpene. The results are shown in Fig. 2B and suggest that MANT-sesquiterpene may bind to TMDs of Pgp. Taken together, these results seem to confirm that MANT-sesquiterpene is a reliable fluorescent derivative to study the mechanism of action of natural sesquiterpenes as Pgp modulators since it retains the activity and mode of action of its natural precursor.

3.2. Pgp does not confer cross-resistance to natural dihydro-β-agarofuran sesquiterpenes because they are not transport substrates of the pump

Using the MTT-based colorimetric cytotoxicity assay, we determined the intrinsic toxicity of three natural sesquiterpenes

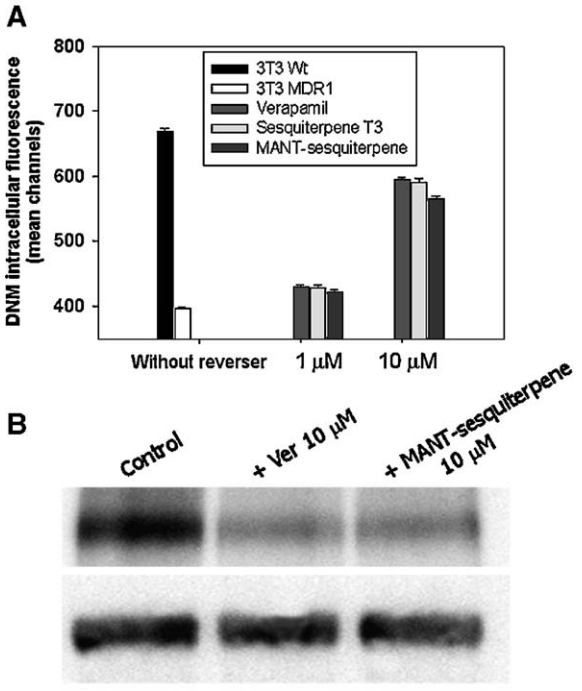

Fig. 2. MANT-sesquiterpene binds to and inhibits Pgp. The anti-Pgp activity of the fluorescent derivative and its natural precursor was established by modulation of DNM accumulation in NIH-3T3 MDR1, monitored by flow cytometry (A). Verapamil was used as positive control of Pgp inhibition for comparison. Results are the mean of two independent experiments, each measurement performed in triplicate. In panel B the displacement is shown of ^{3}H-azidopine binding to the Pgp drug binding-site, as determined by photoaffinity labeling competition experiments. The result is representative of two independent experiments. Upper: fluorography; Lower: immunodetection of Pgp by Western blot using C-219 anti-Pgp monoclonal antibody.

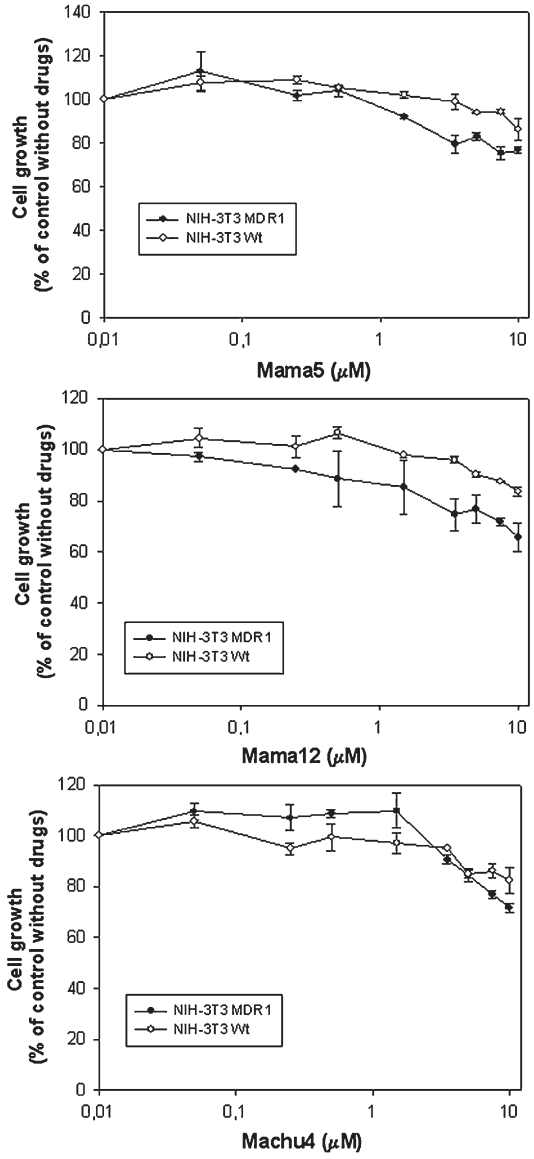

Fig. 3. Cross-resistance to sesquiterpenes due to Pgp expression. Cytotoxicity assays were performed to test the intrinsic toxicity of the three most potent sesquiterpene inhibitors of Pgp available in our laboratory (Mama5, Mama12 and Machu4) against drug-sensitive and NIH-3T3 MDR1 cells. The plots show representative experiments of three different ones, each performed in triplicate.

(Mama5, Mama12 and Machu4). Among those isolated in our lab, these were the most potent compounds to reverse Pgp-dependent MDR [15]. As shown in Fig. 3, none of them was more toxic in drug-sensitive cells than in MDR cells, as would be expected if Pgp conferred cross-resistance to them (i.e., if they were Pgp transport substrates). Strikingly, these natural sesquiterpenes were even slightly more toxic to Pgp-expressing cells than to drug-sensitive cells.

Once we demonstrated that Pgp does not confer cross-resistance to dihydro-β-agarofuran sesquiterpenes, we were interested in demonstrating that the absence of cross-resistance was due to the fact that sesquiterpenes are either poorly transported or not at all by human Pgp. In order to address this issue,

we used MANT-sesquiterpene in accumulation and efflux experiments in Pgp-expressing intact cells. In the accumulation experiments, NIH-3T3 wild-type and MDR1 cells were incubated with either 10 μM MANT-sesquiterpene or 10 μM DNM (a classical Pgp substrate) at increasing times in order to generate time-dependent accumulation curves. As expected (Fig. 4A), DNM readily accumulates in NIH-3T3 wild-type cells but very scantly in MDR1 cells. However, MANT-sesquiterpene (Fig. 4B) accumulates similarly in both cell lines, regardless of Pgp expression. In order to rule out the possibility to overwhelm the ability of Pgp to extrude MANT-sesquiterpene, we repeated the same experiment at 10-fold lower MANT-sesquiterpene concentration (1 μM). In this case, only negligible differences (plots not shown) of MANT-sesquiterpene accumulation were observed between Pgp-expressing and non-expressing cells, similarly to the results shown in Fig. 4A and B.

In efflux experiments, cells were incubated for 2 h with either 10 μM DNM or 10 μM MANT-sesquiterpene. According to the time-dependent accumulation curves described above, 2 h was sufficient to allow the fluorescent probes to reach the maximal accumulation level within the cells. After this incubation step, the fluorophore-containing medium was removed, the cells washed, subsequently incubated in fluorophore-free medium, and the remaining intracellular fluorescence was measured at increasing times. The rate of DNM efflux increased greatly in those cells expressing Pgp at the plasma membrane (Fig. 4C), but the rate of MANT-sesquiterpene efflux was almost unaffected (Fig. 4D). In order to rule out the possibility that the decrease measured in fluorescence was due to intracellular quenching or metabolization of the probes, rather than active efflux, we performed parallel experiments, as described above, measuring the whole fluorescence (intracellular plus extracellular/effluxed) at increasing times, instead of only the remaining intracellular fluorescence. It was observed (data not shown) that both DNM and MANT-sesquiterpene were not quenched intracellularly, but effluxed out of the cells either by active pumping (in the case of DNM) or by passive diffusion down the concentration gradient (in the case of MANT-sesquiterpene).

Finally, to establish that the previous results were not cell-specific but a general phenomenon resulting from sesquiterpene-Pgp interaction, the same experiments were performed in another Pgp-expressing cell line. We chose the line CH^RC5 due to its high Pgp expression levels (up to 5% of total plasma membrane proteins), which would allow us to determine beyond a doubt whether MANT-sesquiterpene was transported or not by the drug efflux pump. The results shown in Fig. 4E and F (DNM and MANT-sesquiterpene, respectively) seem to suggest that MANT-sesquiterpene, although far less than DNM, was actively pumped out by Pgp since the probe accumulated about 20% less in CH^RC5 cells with respect to parental AuxB1 cells. In order to verify that such a finding was not due to differences in the membrane permeability of MANT-sesquiterpene between the parental AuxB1 line and its MDR counterpart, we also performed efflux experiments (Fig. 4G and H), which demonstrated that MANT-sesquiterpene was indeed actively effluxed from Pgp-expressing CH^RC5 cells, although very poorly and far less efficiently than the classical Pgp substrate DNM.

3.3. Intracellular localization of MANT-sesquiterpene in NIH-3T3 cells by fluorescence microscopy

We have demonstrated that MANT-sesquiterpene was a very poor Pgp substrate, whose time-dependent intracellular accumulation was only affected by the drug efflux pump when it is highly over-expressed. In NIH-3T3 MDR1 cells, Pgp expression levels do not reach such high levels, which would explain that even at 1 μM MANT-sesquiterpene, the time-dependent accumulation curves are essentially indistinguishable in both wild-type and MDR1 cells. However, despite not being high enough to affect the intracellular accumulation of MANT-sesquiterpene, Pgp levels in NIH-3T3 cells may be sufficiently high to affect the intracellular distribution pattern of the fluorescent probe. In order to confirm the above, we analysed samples of both cell lines (Pgp expressing and non-expressing NIH-3T3 cells) by fluorescent microscopy after 2-h incubation in the presence of 10 μM MANT-sesquiterpene. The cells were not fixed after the incubation period to avoid any artifacts in the distribution pattern of intracellular fluorescence. No significant differences were found in the distribution pattern between both cell lines: MANT-sesquiterpene enters the cells through the plasma membrane by simple diffusion, and it spreads throughout the entire cytoplasm in a scattered distribution, concentrating mainly in the perinuclear region (possibly the endoplasmic reticulum vesicles) but without staining the cell nucleus (not shown).

3.4. Effect of natural dihydro-β-agarofuran sesquiterpenes on Pgp expression

Previously, we have demonstrated that natural dihydro-β-agarofuran sesquiterpenes bind to TMDs of Pgp and inhibit its drug transport activity in a mixed-type fashion [15]. Moreover, we are reporting in the present work that the pump transports a fluorescent sesquiterpene derivative poorly and does not confer cross-resistance to natural dihydro-β-agarofurans. However, it remained to be determined whether these natural compounds, in addition to inhibiting their activity, affect the expression levels of the protein. In order to clarify the issue, we used a series of murine, hamster and human MDR cells (L1210R, CH^RC5, CH^RB30, CCRF/ADR5000 and CCRF/VCR1000), which over-express Pgp as their major mechanism of multidrug resistance. Each Pgp-expressing cell line was incubated for 3 days in the presence of 3 μM of each natural sesquiterpene (Mama5, Mama12 or Machu4). This concentration was selected for not causing growth inhibition greater than 10% on any of the above cell lines but, however, for being able to reverse resistance to DNM by 60–90% depending on the cell line. Fig. 5 shows the comparative Pgp expression levels in each cell line with/without sesquiterpenes. The protein concentration of all the samples was accurately determined, and equal amounts of each sample were processed in order to allow reliable

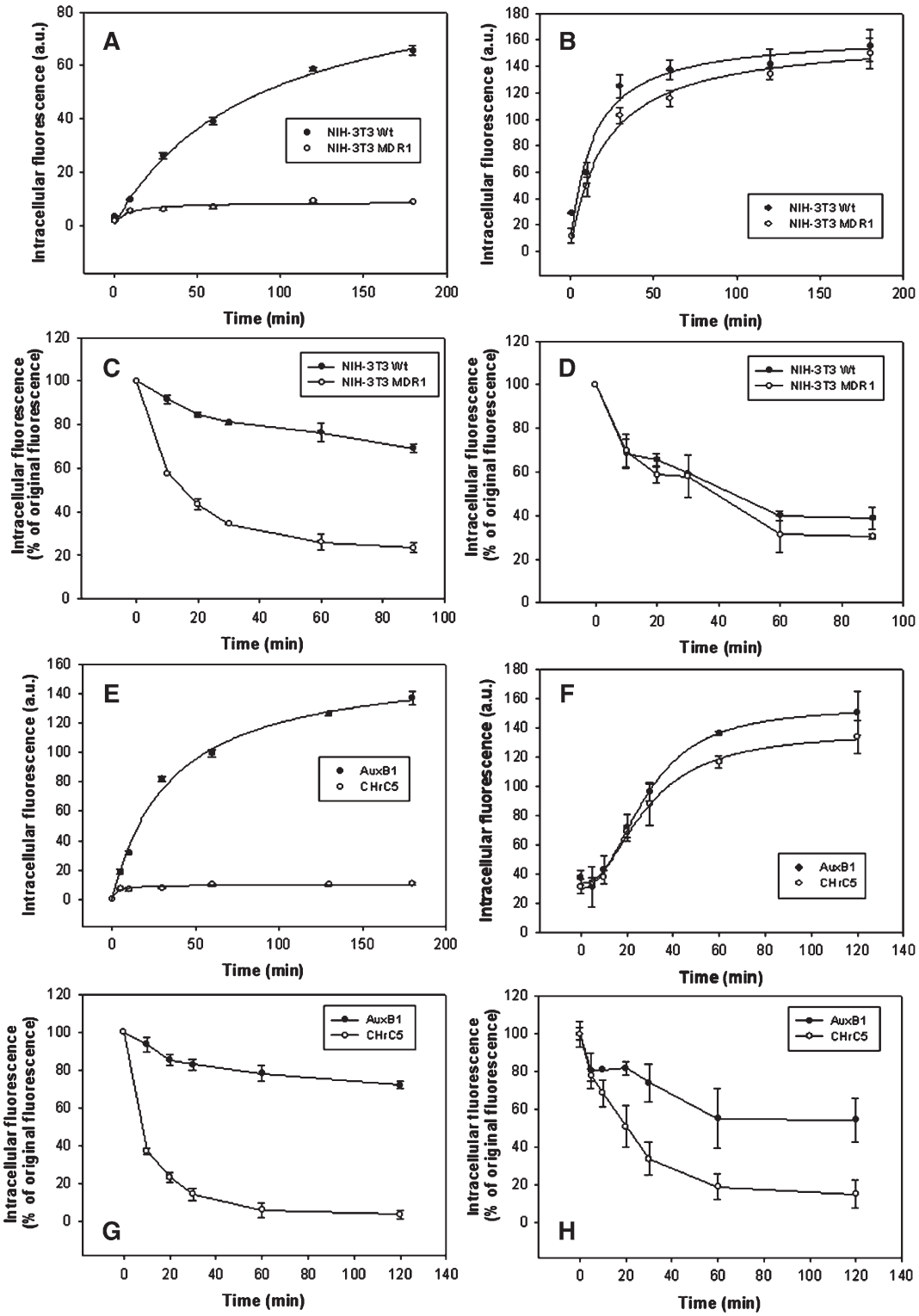

Fig. 4. MANT-sesquiterpene accumulation and efflux experiments in Pgp overexpressing cells. NIH-3T3 MDR1 and drug-sensitive NIH-3T3 cells, as well as AuxB1 and CH^RC5 cells, were incubated with either MANT-sesquiterpene or DNM (10 μM), and the accumulated intracellular fluorescence was measured at increasing times. In parallel experiments, the same cell lines were allowed to accumulate each fluorescent probe over 2 h, then washed and further incubated in probe-free medium; the remaining intracellular fluorescence was then measured at increasing times after washing (efflux experiments). The right column of plots represents experiments performed using MANT-sesquiterpene as a probe for Pgp-mediated transport, and the left one shows those experiments using DNM for comparison as a classical Pgp substrate. Accumulation (A, B, E and F) and efflux experiments (C, D, G and H) were carried out using NIH-3T3 MDR1 cells (A, B, C and D) and CH^RC5 cells (E, F, G and H). Each plot is a representative experiment of two different ones, each performed in triplicate.

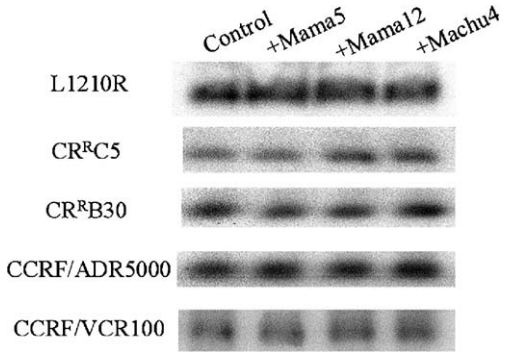

Fig. 5. Influence of sesquiterpenes on Pgp expression. Pgp expression levels in different MDR cell lines were determined by Western blot and subsequent immunodetection of the multidrug transporter after 3 days' incubation of each cell line with a subtoxic concentration (3 μM) of the three most potent anti-Pgp sesquiterpenes described in our laboratory. After this incubation period, cells were homogenated and the entire membrane fraction was recovered. The protein concentration was accurately determined in each sample, and equal amounts of membrane proteins were resolved in SDS-PAGE and processed as described in Materials and methods.

comparisons of Pgp levels. No significant alteration of Pgp expression levels was detected.

3.5. Duration of the reversal effect of natural sesquiterpenes in NIH-3T3 MDR1 cells

The time-dependent curve of MANT-sesquiterpene efflux from NIH-3T3 cells (Fig. 4D) showed that the probe, in the initial stage of efflux, left the cells quite fast by simple diffusion down the concentration gradient; however, after 1 h, the efflux rate slowed down dramatically, and the intracellular concentration of the probe remained stable at levels 30–40% of the original fluorescent signal, decreasing then only very slowly. This suggested that sesquiterpenes exist inside the cells in two forms, one that could be free in the cytoplasm and diffuses fast from the cells (initial stage of rapid efflux), and another form tightly bound to its intracellular target(s), or sequestered inside vesicles (later stage of very slow efflux). This implies that sesquiterpenes, until complete removal, may remain for a considerable time inside the cells exerting their pharmacological action as Pgp inhibitors. In order to test this assumption, we incubated NIH-3T3 MDR1 cells with 10 μM of Machu4 (the most potent sesquiterpene Pgp inhibitor) for 2 h, in order to load cells with the natural sesquiterpene. Afterwards, cells were washed and the ability of Pgp to actively extrude DNM was tested in DNM accumulation experiments at increasing times after washing the sesquiterpene from the cell culture medium. In this situation, only the amount of sesquiterpene remaining inside the cells could exert any measurable Pgp inhibition. As controls, the DNM-extruding activity of Pgp was also determined in the absence (100% Pgp activity) and in the continuous presence (complete inhibition of Pgp activity) of 10 μM Machu4. Fig. 6 shows the time-dependent DNM accumulation curves in the continuous presence, and absence of Machu4, and after its removal from the cell culture medium. It is clear that, after washing, the sesquiterpene is still able to block the Pgp-dependent DNM extrusion for a considerable time. Plotting the DNM accumulation level at a fixed time (20 min) against the time elapsed after Machu4 removal from the extracellular medium (plot not shown), the half-life of Machu4 activity inside the cells was estimated to be around 30 h.

3.6. Ability of MRP1, MRP2 and BCRP to transport MANT-sesquiterpene

In order to explore the putative role of other human MDR efflux pumps as potential transporters of dihydro-β-agarofuran sesquiterpenes, the same MANT-sesquiterpene accumulation experiments performed in Pgp-expressing cells were also performed in cells over-expressing human MRP1, MRP2 or BCRP multidrug efflux pumps. MANT-sesquiterpene was also used at 10 μM, similarly to experiments in Pgp-expressing cells, and a well-known fluorescent substrate for MRP1, MRP2 and BCRP was tested in parallel at a relevant concentration for comparison to MANT-sesquiterpene. The results of this set of experiments are shown in Fig. 7. The probe calcein-AM was actively effluxed by both MRP1 and MRP2 pumps (Fig. 7A and C), but MANT-sesquiterpene was not (Fig. 7B and D). Similarly, Hoechst 33342 was effluxed as expected for a BCRP substrate (Fig. 7E), but MANT-sesquiterpene was not (Fig. 7F). Therefore, these results suggest that none of the major human multidrug ABC transporters plays a significant role in the active transport of natural dihydro-β-agarofuran sesquiterpenes.

3.7. Synergistic and co-operative interactions between natural sesquiterpenes and classical modulators to bring about Pgp inhibition

Table 1 shows, on one side, the effects (observed as K_i and Hill number variations) of fixed Mama12 concentrations

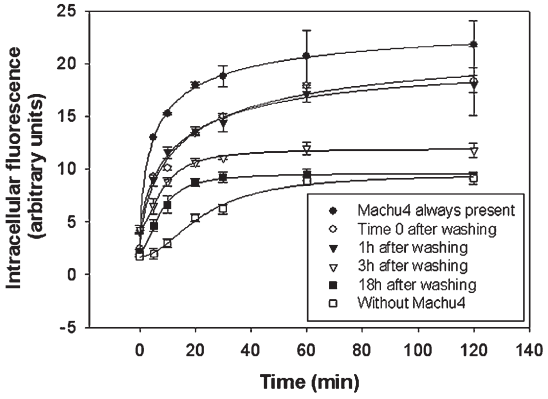

Fig. 6. Duration of the reversal effect of sesquiterpenes. NIH-3T3 MDR1 cells were incubated with sesquiterpene Machu4 10 μM for 2 h, and then the cells were washed to eliminate any trace of extracellular sesquiterpene. Then, the ability of the intracellularly accumulated Machu4 to block Pgp drug transport activity was estimated by measuring the time-dependent accumulation of DNM (10 μM) in the continuous presence/absence of Machu4, or at different times after washing. The plot shows a representative experiment of two independent DNM accumulation experiments, each performed in triplicate.

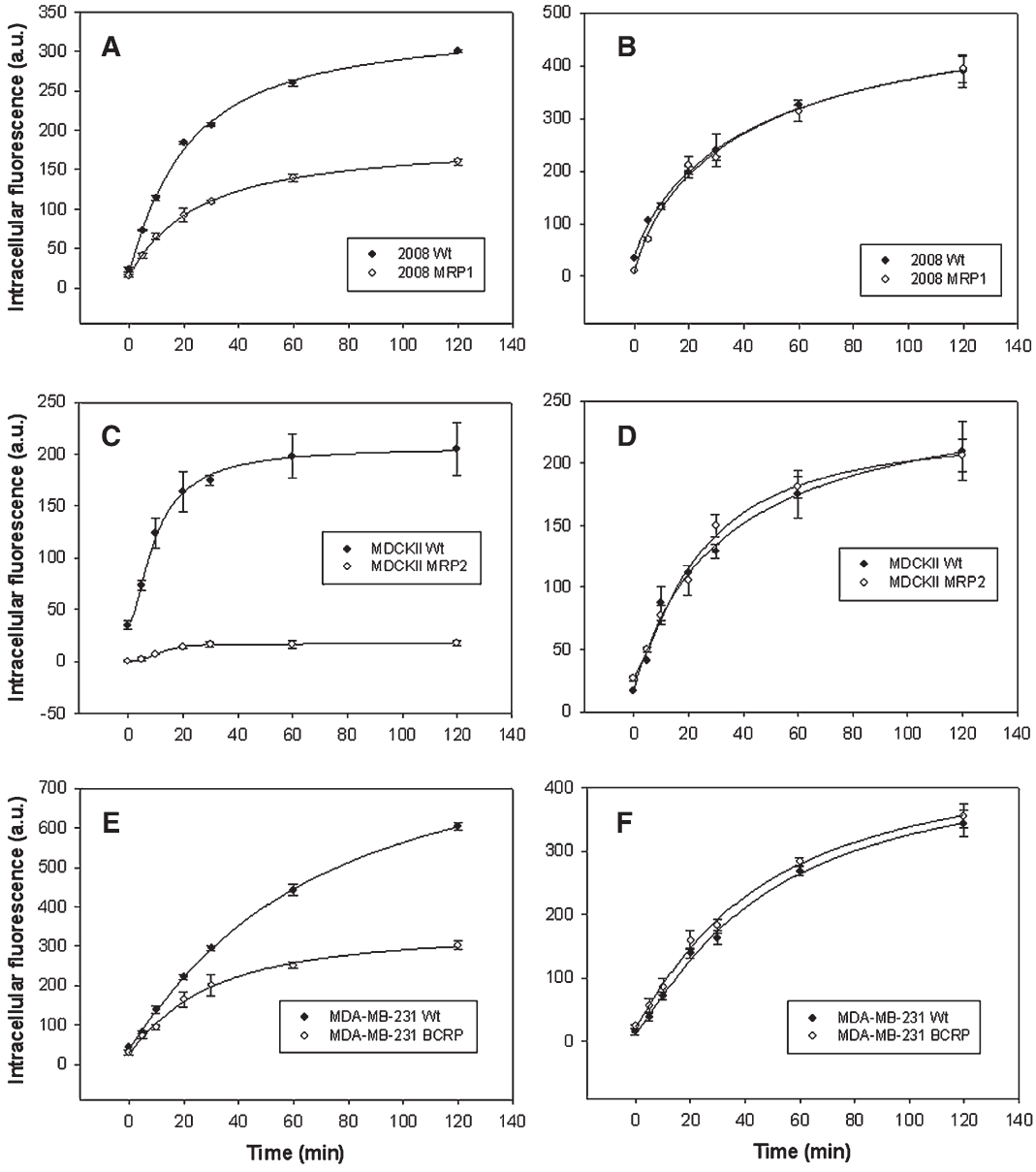

Fig. 7. Ability of MRP1, MRP2 and BCRP multidrug transporters to efflux MANT-sesquiterpene. Cell lines overexpressing the human multidrug transporters MRP1 (B), MRP2 (D) and BCRP (F), and their drug-sensitive parental counterparts, were incubated with 10 μM MANT-sesquiterpene. The accumulated fluorescence was measured at increasing times. 1 μM calcein-AM for MRP1 (A) and MRP2 (C), or 1 μM Hoechst 33342 for BCRP (E) were used as control probes for measuring drug transport mediated by each of these multi-drug transporters.

(ranging from 0 to 1–2 μM) on the Pgp inhibition caused by increasing concentrations of five different classical Pgp modulators (verapamil, VNB, CsA, GF120918 and PRG). On the other side of the table is shown, conversely, the effect of increasing fixed concentration of the five classical modulators mentioned earlier on K_i and the Hill number of sesquiterpene Mama12 as a blocker of Pgp-dependent DNM efflux in intact cells. The values of the Hill number and K_i for Pgp-mediated DNM transport inhibition were computed from curves fitted to the experimental data points accordingly to Eq. (1) or Eq. (2) (see Materials and methods), depending on the case. These values are expressed as means ± S.E. ($P < 0.001$) of two different experiments for each pair of modulators, performed in triplicate.

The intrinsic K_i of verapamil decreases slightly with increasing Mama12 concentrations, rendering the Hill number unaffected. However, K_i of Mama12 increases in the presence of increasing verapamil concentrations, and its Hill number decreases. An explanation of this finding is that Mama12 could have 2 different binding sites, of high and low affinity, respectively, within Pgp that are not coupled allosterically, since the Hill number for Mama12-mediated inhibition of Pgp transport activity is not much higher than 1. The binding of Mama12 to its high affinity binding site would decrease verapamil K_i (non-competitive interaction), but the Mama12 low affinity-binding site and the verapamil-binding site could interact physically (competitively), causing the Mama12 Hill

Table 1
Interactions between pairs of Pgp modulators when inhibiting Pgp-mediated DNM transport in intact cells

2nd modulator	μM	Hill number	K_i (μM)
Verapamil			
Mama12	0	1.07±0.12	1.17±0.13
	0.25	1.11±0.19	1.08±0.17
	0.5	1.10±0.29	0.67±0.12
	1	1.16±0.34	0.34±0.23
CsA			
Mama12	0	1.49±0.19	0.645±0.031
	0.125	1.43±0.22	0.708±0.071
	0.25	1.36±0.24	0.750±0.128
	0.5	1.19±0.12	0.891±0.093
VNB			
Mama12	0	2.73±0.41	26.14±2.62
	0.25	2.51±0.32	24.86±3.15
	0.5	2.64±0.36	17.66±0.72
	1	2.41±0.38	12.31±1.65
GF120918			
Mama12	0	1.52±0.16	0.113±0.008
	0.125	1.71±0.28	0.071±0.007
	0.25	1.44±0.34	0.062±0.009
	0.5	1.49±0.29	0.045±0.005
PRG			
Mama12	0	2.22±0.24	41.73±7.88
	0.25	2.15±0.14	37.64±2.42
	0.5	2.39±0.22	27.76±1.83
	1	2.30±0.33	15.61±2.76
Mama12			
Verapamil	0	1.06±0.08	0.70±0.11
	1	0.86±0.07	0.83±0.15
	2	0.68±0.05	1.02±0.22
	3	0.65±0.05	1.93±0.35
CsA	0	1.16±0.13	0.65±0.04
	0.125	1.08±0.07	0.73±0.03
	0.25	1.10±0.10	0.70±0.03
	0.5	1.09±0.04	0.74±0.05
VNB	0	1.18±0.10	0.77±0.08
	5	1.17±0.09	0.74±0.06
	10	1.12±0.05	0.66±0.07
	20	1.07±0.12	0.42±0.09
GF120918	0	1.25±0.13	0.76±0.06
	0.062	1.23±0.23	0.70±0.08
	0.125	1.17±0.19	0.47±0.02
	0.25	1.27±0.14	0.23±0.07
PRG	0	1.28±0.12	0.96±0.07
	10	1.32±0.18	2.13±0.38
	20	1.91±0.30	1.46±0.18
	30	2.42±0.37	1.36±0.19

It is shown how increasing fixed concentrations of a second modulator modify the kinetic parameters of Pgp inhibition caused by a first modulator. By a simple analysis of these data, it is possible to determine the type of interaction (competitive, non-competitive, synergistic) that exist between a given pair of Pgp modulators when combined to bring about Pgp inhibition.

number to drop and its intrinsic K_i to increase. This notion of the existence of two different sesquiterpene binding sites within Pgp is further supported by the fact that sesquiterpenes modulate Pgp ATPase activity in a biphasic way, stimulating ATPase at concentrations lower than 1 μM and then inhibiting it at higher concentrations [15].

In turn, Mama12 competitively interacts with CsA since both modulators mutually increase their respective K_i. However, the Mama12 Hill number, affected by the presence of verapamil, remains unaffected by CsA. This suggests that CsA and verapamil, although interacting competitively, and also competitively with respect to Mama12, do not interact with Mama12 in exactly the same way. It is possible that CsA, given its huge molecular volume, overlaps with both the high- and low-affinity Mama12-binding sites and affects them indistinctively, but verapamil is able only to overlap with one binding site, presumably the low-affinity one.

Mama12 and VNB seem to interact synergistically (non-competitively), collaborating with each other to bring about Pgp inhibition, since they mutually cause a decrease in their respective K_i values without significantly affecting their respective Hill numbers. In the same way, Mama12 and GF120918 also interact in a synergistic manner, mutually boosting their effects as Pgp-mediated DNM transport inhibitors.

Regarding the pair of modulators PRG and Mama12, when PRG acts as the second modulator, two different effects on Mama12 kinetic parameters can be distinguished: at PRG concentrations lower than 10 μM, Mama12 K_i increases and the Hill number remains constant, but at higher PRG concentrations (up to 30 μM), Mama12 K_i decreases and the Hill number increases. This would suggest the existence of two different PRG-binding sites of high and low affinity, each one interacting with Mama12 binding in a different manner: on one hand, a competitive interaction would take place between the high affinity PRG-binding site and Mama12-binding site(s) and, on the other hand, a non-competitive interaction between Mama12-binding site(s) and the low affinity PRG-binding site. However, much care should be taken when interpreting these results since PRG is quite an unusual Pgp modulator. It is known that PRG indeed possesses two different binding sites within Pgp, one of high affinity located at the TMDs (affinity constant lower than 10 μM, as shown in [29]), and another of lower affinity located at the NBDs, with an affinity constant around 18-53 μM, depending on the tested steroid [30]. PRG, at high concentrations, inhibits Pgp drug transport activity, but at concentrations around 10 μM or lower, it has the unusual property among Pgp modulators to be able to stimulate the Pgp-mediated transport of those substrates that bind to either the H site (for Hoechst 33342 and colchicine) or to the R site (for rhodamine 123 and anthracyclines) within the TMDs in a positive allosteric manner [31]. DNM is an anthracycline and, hence, it is expected to bind to the R site, and its transport to be stimulated accordingly by PRG. We have indeed observed such DNM transport stimulation by PRG (not shown), but at concentrations higher than 10 μM this stimulatory effect of PRG disappeared, and only its inhibitory effect remained. The presence of Mama12 up to 1 μM was unable to preclude this phenomenon of DNM transport stimulation by PRG, suggesting that both PRG- and Mama12-binding sites within TMDs are not overlapping physically, and that the apparent increase in Mama12 K_i caused by PRG up to 10 μM (as shown in Table 1) is the consequence of the DNM

transport stimulation caused by PRG in this concentration range, rather than a competitive interaction between PRG and Mama12. A more detailed kinetic analysis was performed of the combination of PRG up to 10 μM (stimulating concentration range) with different fixed concentrations of Mama12, by using the "Exploratory Enzyme Kinetics" application of SigmaPlot 2000 software for Windows. This revealed, indeed, a mixed-type interaction between both modulators, which argues against physical interactions between their respective binding sites within TMDs. On the other hand, the observed drop in Mama12 K_i caused by PRG concentrations higher than 10 μM should be due to the fact that the binding of PRG to its low affinity-binding site at the NBDs causes a conformational change, as previously described [30]. Such a change should then transmit to TMDs and modify the Mama12-binding site(s) in a sense that increases their affinity for Mama12 and also the Hill number to values significantly equal to 2, coupling both sites allosterically to bring about Pgp inhibition.

In turn, fixed Mama12 concentrations, when combined with increasing PRG concentrations over 10 μM (inhibitory range), induces a decrease in PRG K_i without affecting its Hill number, suggesting non-competitive interactions, as was also suggested above. The Mama12-binding site(s) should not overlap with PRG-binding site within NBDs since dihydro-β-agarofuran sesquiterpenes do not interact physically with that Pgp domain [15], which further supports the result discussed earlier. Mama12 binding to TMDs produces a conformational change in Pgp that, despite its little magnitude (as shown in [15]), should transmit to the NBDs and cause the steroids-binding site to increase its affinity for PRG.

4. Discussion

In our previous work [15], we demonstrated that natural dihydro-β-agarofuran sesquiterpenes from *Celastraceae* were able to reverse potently cellular MDR by blocking specifically human Pgp as mixed-type inhibitors of its drug transport activity. Moreover, we also showed that these natural compounds did not affect the activity of MRP1, MRP2 and BCRP, providing evidence of their specificity of action. In the present work, by using a newly synthesized sesquiterpene fluorescent derivative (MANT-sesquiterpene) that possesses the anti-Pgp activity of its natural precursor, we have proceeded to further characterize the molecular mechanism of action of dihydro-β-agarofuran as Pgp-inhibitors, supporting the findings of our previous work. We have demonstrated, first of all, that Pgp does not confer cross-resistance to natural sesquiterpenes and, secondly, that this multidrug efflux pump, compared to classical Pgp substrates such as DNM, transports MANT-sesquiterpene almost insignificantly. In fact, such net transport mediated by Pgp across the plasma membrane is only apparent in cells that highly over-express the transporter, and only when the efflux of the probe, rather than its accumulation, is measured. This new finding, firstly, supports our previous finding that showed dihydro-β-agarofuran sesquiterpenes to be potent Pgp inhibitors since it is well known that good Pgp substrates are usually poor inhibitors and vice versa [32]. And secondly, it lessens the possibility that Pgp contributes significantly to sesquiterpenes transport in vivo since no tissue or tumour has been shown to over-express Pgp up to the levels (5% of total membrane proteins) necessary to observe any signal of net Pgp-mediated sesquiterpene transport. Moreover, neither MRP1/MRP2 nor BCRP efflux pumps are able to transport MANT-sesquiterpene across the plasma membrane. Therefore, none of the ABC multidrug efflux pumps implicated in cancer MDR may be considered to be a resistance factor against natural sesquiterpenes and, presumably, they would also be unable to affect their intestinal absorption and biodistribution. That is an additional requirement, along with potency and specificity of action, which any ideal MDR modulator should satisfy.

We have also observed that the intracellular distribution of MANT-sesquiterpene is not only restricted to the plasma membrane (the final destination of functional Pgp in the majority of cells), but the probe is also able to get inside the cells and spread intracellularly, giving a scattered pattern of intracellular fluorescence that concentrates mainly on the perinuclear region of the cytoplasm. This suggests that the probe accumulates in membrane vesicles of Golgi and endoplasmic reticulum (ER), and that natural sesquiterpenes potentially inhibit the activity not only of Pgp at the plasma membrane, but also the newly synthesized pump, at ER level and vesicles of the secretory pathway, along its entire path towards the plasma membrane. The fact that sesquiterpenes do not seem to be inactivated by intracellular metabolism (not shown), and the long half-life of its intracellular activity (up to 30 h) further support its potential ability to inhibit efficiently Pgp for a long time both at the plasma membrane and in intracellular compartments. The reversal efficiency and duration of the anti-Pgp activity of sesquiterpenes within cells would be of great importance for the clinical reversion of MDR in tumours, due to the fact that inhibition of Pgp activity in 100% of Pgp-expressing cells would avoid the risk of selecting MDR clones that would expand and repopulate those tumours that were initially responsive to chemotherapy. Moreover, it has been observed that Pgp, along with its drug transport-related role in MDR, also seems to exert anti-apoptotic activities due to its ability to modulate ceramide-mediated apoptosis. It is presumed that Pgp is able to do so by translocating both sphingomyelin from the inner to the outer leaflet of plasma membrane [33,34], and glucosyl-ceramide [35] from the cytoplasm to the Golgi lumen in human breast cancer cells [36] and acute myeloid leukemia cells [37]. Moreover, it has also been shown that Pgp inhibits FAS-induced caspase-8 activation in an ATP hydrolysis-dependent fashion [38]. Therefore, the potential clinical use of sesquiterpenes as inhibitors of Pgp activity would not only consist in reversing classical MDR, but also in restoring sensitivity to FAS- and ceramide-dependent apoptosis in those malignancies in which Pgp plays a well-defined anti-apoptotic role.

We have also shown, by using a battery of Pgp-over-expressing cells, that none of the most potent natural sesquiterpenes is able to alter Pgp expression levels in long-term incubations. The over-expression of Pgp in cancer cells exposed

to chemotherapeutics is a typical response of cells to stress signals [39]. Since stress is able to trigger many cellular responses simultaneously, it has been suggested that Pgp overexpression is only a part of a concerted, more global stress-induced response for cell survival, orchestrated by orphan nuclear receptors [40,41]. The fact that natural sesquiterpenes inhibit Pgp activity but do not induce its expression would suggest, first, that they may not interfere with the *mdr1* transcription, translation or protein folding process and, second, that they may not interact with orphan nuclear receptors. The latter assumption is of particular importance since this presumed property, if verified, would imply that natural sesquiterpenes behave as "clean" MDR reversers that would not trigger global protective responses in cancers, such as those involving changes in the expression of other stress proteins also involved in MDR along with Pgp (glutathione-*S*-transferase, cytochrome P450 enzymes, DNA-repairing enzymes, etc.).

Finally, the kinetic analysis of the interactions between the natural sesquiterpene Mama12 and classical Pgp modulators to inhibit Pgp-dependent DNM transport confirmed the TMDs of the pump as the location for dihydro-β-agarofurans binding site (s) within the protein. In addition, this analysis also allowed us to find examples of both competitive and non-competitive interactions between Mama12 and classical modulators, which were consistent with previous information about the way in which classical modulators interact with one another in pairs to produce inhibition of Pgp. In brief, Mama12 possesses two binding sites within TMDs of high and low affinity, and at least one of them overlaps with verapamil and CsA binding sites, presumably sharing with them the so-called pharmacophore 1 described in [42]. Moreover, Mama12 interacts non-competitively with both VNB (which occupies the so-called pharmacophore 2) and the PRG high and low affinity binding-sites. The fact that Mama12 does not compete with VNB, which is presumed to bind to the Pgp H and R sites for drug transport [31], is in agreement with our previous results [15] that showed Mama12 to be a mixed-type inhibitor of Pgp-mediated tetramethylrosamine transport (a dye known to bind to the R site). Finally, Mama12 interacts synergistically with GF120918 (elacridar), mutually increasing their respective potencies to bring about Pgp inhibition.

In summary, all these findings suggest that dihydro-β-agarofurans are a promising new family of Pgp inhibitors that may be safely and usefully used in clinical practice. Moreover, its privileged scaffold would allow the rational design of new structures even more potent and specific for Pgp. All the information that we have obtained about the molecular mechanism of action of dihydro-β-agarofurans will help us on such rational design and, in fact, future work in our laboratories will focus on that task using QSAR-based approaches.

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